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13. ABSTRACT (Maximum 200 Words)

Cadherin-11 is unique amongst cadherins in that it is exists as two alternatively spliced forms that are expressed together in the same cell. In year 1 of this grant we showed that the presence of the cadherin-11 splice variant promotes invasion of cadherin-11 positive breast cancer cells, perhaps by promoting cell-ECM interactions. In year two of the grant we have tested the hypothesis that inhibition of cadherin-11 function using proprietary small molecule cadherin-11 inhibitors influences the ability of cadherin-11-expressing cells to invade in vitro. Our data show that a particular class of inhibitors designed to block the ability of cadherin-11 to interact with the extracellular matrix does indeed inhibit cell invasion. Other molecules designed to disrupt the cell-cell adhesive function of cadherin-11 did not affect cell invasion. In other studies we have generated cell lines expressing siRNA directed at both cadherin-11 and cadherin-11 variant.

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Introduction

Previously, loss of expression or function of the epithelial cell-cell adhesion molecule E-cadherin was found to be associated with a loss of epithelial phenotype and with a gain of invasiveness in a number of cancers including breast cancer (Pishvaian *et al.*, 1999). In contrast expression of cadherin-11 and its variant form were found to be expressed in the most invasive breast cancer cell lines but were not detected in non-invasive cell lines (Pishvaian *et al.*, 1999). Further, we showed in the last report that expression of the cadherin-11 splice variant promotes invasion of cadherin-11-positive breast cancer cells (Feltes *et al.*, 2002). Hence cadherin-11 expression was suggested to be correlated with the invasive phenotype in cancer cells and could potentially serve as a predictor of the invasive and metastatic phenotype. The work described in this annual report was designed to test the ability of small molecule cadherin-11 inhibitors influence cell invasion as described in the original statement of work. To this end, proprietary small molecule cadherin-11 inhibitors obtained form Adherex Technologies were tested for their potential to inhibit invasion of cadherin-11 expressing MDA-MB-231 cells *in vitro*. Boyden chamber assays were performed and results showed a number of peptides to efficiently inhibit cell invasion.

Materials and Methods and Results:

Materials and Methods

Boyden chamber invasion assay

Day 1

Matrigel solution was diluted in H_2O in a 10cm diameter tissue culture plate to a final concentration of 20μg/ml. An 8μm porous membrane was submerged top-side down in the matrigel solution and was left at 4°C overnight. A 175cm^2 flask of low passage number MDA-MB-231 cells was serum-starved overnight.

Day 2

Following coating with matrigel the membrane was air-dried using appropriate clamps for at least 3 hours. The membrane must not touch any surface during the drying process. The Boyden chamber was rinsed 3 times with ddH_2O and subsequently dried in the tissue culture hood.

Before assembly of Boyden apparatus, the appropriate peptide or control solutions were prepared. Twice the required concentration was diluted into 250ul of serum-free DMEM tissue culture medium. Subsequently, the serum-starved MDA-MB-231 cells were washed once with 1X PBS and trypsinised. The cell number per ml was determined using a Coulter counter. As each Boyden chamber well required 10000 cells, 100000 cells were needed for a final volume of 500μl for each treatment. Therefore, trypsinised cells were resuspended at a concentration of 100000 cells per 250μl serum-free DMEM tissue culture medium and subsequently added to the previously prepared 250ul of peptide solutions.

Next, the bottom chamber of the Boyden apparatus was loaded with the chemoattractant (15% FBS containing DMEM medium). It was important to create a meniscus that ensures saturation of the membrane with chemoattractant but prevents the chemoattractant from overflowing into neighbouring wells. Following the addition of chemoattractant, the air-dried membrane was placed top-side up onto the bottom chamber. Then the upper chamber of the Boyden apparatus, with the rubber gasket flush and securely attached to the bottom of the upper chamber, was secured onto the bottom chamber using screws **(Fig. 1)**.

Upon completed assembly of the Boyden apparatus, 50μl of the pre-prepared peptide-cell or control-cell solutions were loaded into the wells of the top chamber. Each peptide or control treatment was loaded in triplicate and a positive control (no treatment) and negative control (no chemoattractant) were included. Care was taken to avoid air-bubbles that would inhibit invasion of cells. Cells were left to invade for 16 hours at 37°C.

Following the incubation time, 10ml of DiffQuik fixative were aliquoted into a 10cm diameter tissue culture plate. The Boyden apparatus was carefully disassembled so that the membrane would remain attached to the inverted top chamber. Using two forceps the membrane was moved from the inverted top chamber into the fixative solution. The membrane was incubated in fixative for 5 minutes at room temperature. After incubation the initially bottom-side up membrane was inverted. The fixative was subsequently poured out and the membrane was incubated in 10ml of cytoplasm staining solution (DiffQuik) for 3 minutes at room temperature. After removal of dye the membrane was submerged in nuclei staining solution (DiffQuik) for 5 minutes at room temperature. Finally, the membrane was washed twice in ddH_2O. The membrane was kept submerged in ddH_2O while cells was carefully swiped of the top-side of the membrane using cotton tip. The membrane was dried using the appropriate clamps as before. Meanwhile the apparatus was rinsed with distilled water.

The air-dried membrane was dessicated in xylenes for at least 90 minutes before being mounted on a glass slide using Cytoseal 60. The membrane was covered with coverslips and left to dry.

For analysis, pictures of each cell invasion spot were taken using an Olympus Vanox microscope at x2.5 magnification. A focusing reticle served to determine the centre of each cell invasion spot. The pictures of the cells were further analysed using the Metamorph analysis program to determine the number of stained nuclei, hence number of cells, within each picture. Tools within the Metamorph program were employed to ensure discrimination between pores and nuclei.

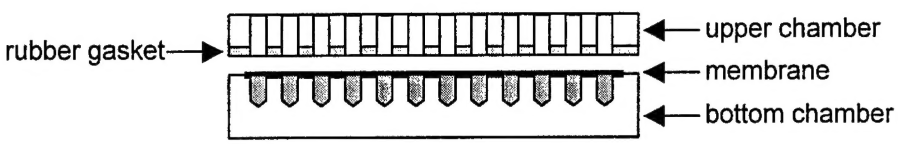

Fig.1: Diagram of assembly of Boyden chamber apparatus.

Results and Discussion

Name	50% inhibition
ADH92	>1mg/ml
ADH93	~ 0.6mg/ml
ADH114	~0.6mg/ml
ADH113	> 1mg/ml
ADH549	~0.6mg/ml
ADH548	N/A
ADH243	not supplied

Table 1: Summary of cadherin-11 inhibitory peptides. Level of activity is expressed as peptide concentration resulting in approximately 50% inhibition of cell invasion

As shown in **Fig.2**, ADH93 inhibited MDA-MB-231 cell invasion through a matrigel- coated membrane at peptide concentrations of ≥0.6mg/ml. In contrast, ADH92 did not have an observed effect on cell invasion.

The effect of ADH114 and ADH113 are shown in **Fig. 3**. A marked decrease in the number of cells invading the matrigel-coated membrane was observed using ADH114. An approximately 50% reduction in cell invasion occurred at 0.6mg/ml peptide concentration and was maintained at higher concentrations. On the contrary, ADH113 did not result in a significant decrease in cell invasion.

The range of peptide concentrations tested revealed ADH93 and ADH114 to inhibit 50% of cell invasion at ~0.6mg/ml peptide concentration. Concentrations above 1mg/ml were not analysed, as the majority of peptides was not soluble at higher concentrations (**table 1**).

Analysis of ADH549 and its inhibitory effect on MDA-MB-231 cell invasion, gave a similar result to ADH93 and ADH114. Again, an approximately 50% reduction in cell invasion was observed at ≥0.6mg/ml peptide concentration (**Fig. 4**). To support the experimental result, pictures of the cell invasion spots on the membrane were taken. These clearly show the marked reduction in cell invasion between 0.4 and 0.8mg/ml peptide concentration (**Fig. 4**).

Analysis of ADH548 gave no results as the peptide required to be dissolved in ddH2O which subsequently interfered with cell viability at peptide concentrations higher than 0.1 mg/ml. An answer to the above problem would be the use of phosphate-buffered saline solution or to improve the solubility of the peptide in serum-free DMEM tissue culture medium.

Finally, an investigation into the effect of ADH549 on morphology of MDA-MB-231 cells, revealed cells at the edges of colonies to adopt a thin-elongated shape before eventually disattaching from neighbouring cells and the coverslip. Cells had been exposed to 1mg/ml of ADH549 and the effect was more pronounced with increasing time.

In conclusion, three peptides, namely ADH93, ADH114 and ADH549 were shown to cause a decrease in cell invasion of MDA-MB-231 cells at ≥0.6mg/ml concentrations. In contrast, ADH92 and ADH113 did not reveal any effect on cell invasion. In addition, changes in cell morphology were observed when exposing MDA-MB-231 cells to 1mg/ml ADH549 over a time-span of 16 hours.

Therefore, ADH93, ADH114 and ADH549, in particular, may be recommended for further experimental analysis.

Generation of MDA-231 cells expressing cadherin-11 siRNA:

As proposed in the original statement of work we have generated cell lines stably expressing cadherin-11 siRNA. This work is ongoing but our data so far show that we have identified cadherin-11 and cadherin-11 variant sequences that can be successfully targeted by transiently transfected siRNA. Cell lines stably expressing these siRNAs are presently being generated.

Generation of an antibody directed against cadherin-11 variant:

In the last report we described the production of an antibody directed against unique sequences found in the cadherin-11 variant form. We showed that this antibody is specific for the variant form of cadherin-11 and does not recognize cadherin-11 itself; the antibody is now being marketed by Zymed.

Key Research Accomplishments:

1. Demonstration that cadherin-11 blocking small molecules can inhibit the invasion of cadherin-11-expressing cells.

2. Generation of cadherin-11 siRNA-expressing cell lines:

Reportable Outcomes:

An alternatively spliced cadherin-11 regulates human breast cancer cell invasion. Paper published in Cancer Research (Feltes, C.M., Kudo, A., Blaschuk, O., Byers, S.W. (2002) An alternatively spliced cadherin-11 enhances human breast cancer cell invasion. *Cancer Res.*, **62**, 6688-6697)

Compounds and Methods for Inhibiting cancer metastasis. Patent awarded July 15th 2003 (note that OB-cadherin is another name for cadherin-11)

United States Patent *6,593,297*
Blaschuk , et al. **July 15, 2003**

Compounds and methods for inhibiting cancer metastasis

Abstract

Agents for inhibiting cancer metastasis are provided. The methods comprise administering to a patient an antimetastatic agent that comprises one or more of: (a) a peptide sequence that is at least 50% identical to an OB-cadherin CAR sequence; (b) a non-peptide mimetic of an OB-cadherin CAR sequence; (c) a substance, such as an antibody or antigen-binding fragment thereof, that specifically binds an OB-cadherin CAR sequence; and/or (d) a polynucleotide encoding a polypeptide that comprises an OB-cadherin CAR sequence or analogue thereof.

Inventors: **Blaschuk; Orest W.** (Westmount, CA); **Symonds; James Matthew** (Ottawa, CA); **Byers; Stephen** (Washington, DC); **Gour; Barbara J.** (Kemptville, CA)

Assignee: **Adherex Technologies, Inc.** (Ottawa, CA)

Appl. No.: **264516**

Filed: **March 8, 1999**

Conclusions:

Our data show that a particular class of inhibitors designed to block the ability of cadherin-11 to interact with the extracellular matrix does indeed inhibit cell invasion. Other molecules designed to disrupt the cell-cell adhesive function of cadherin-11 did not affect cell invasion. In other studies we have generated cell lines expressing siRNA directed at both cadherin-11 and cadherin-11 variant. These data strongly indicate that inhibition of the ability of cadherin-11 to interact with the ECM blocks cell invasion. The demonstration that small molecule inhibitors can effectively block this important function of cadherin-11 bodes well for the development of drugs that can inhibit the ability of cadherin-11 expressing cells to metastasize.

References

Feltes, C.M., Kudo, A., Blaschuk, O., Byers, S.W. (2002) An alternatively spliced cadherin-11 enhances human breast cancer cell invasion. *Cancer Res.*, **62**, 6688-6697.

Pishvaian, M.J., Feltes, C.M., Thompson, P., Bussemakers, M.J., Schalken, J.A. et al. (1999) Cadherin-11 is expressed in invasive breast cancer cell lines. *Cancer Res.*, **59**, 947-952.

Appendix:

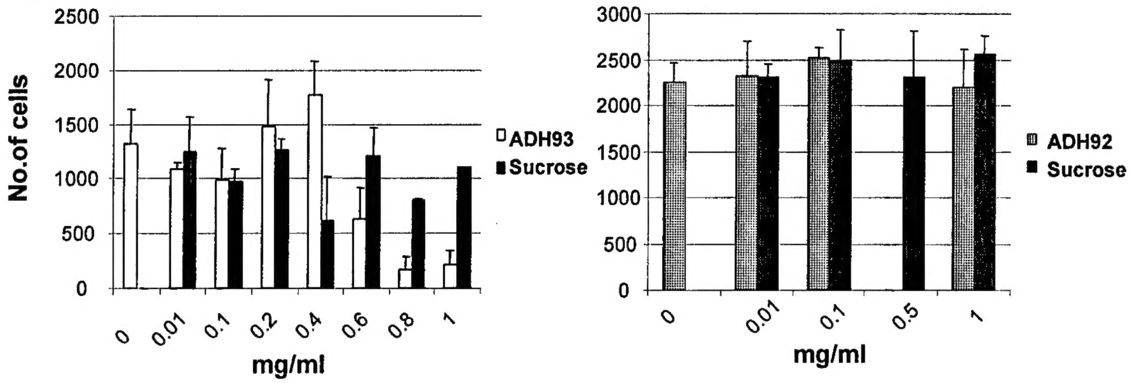

Fig. 2: Inhibition of MDA-MB-231 cell invasion through matrigel-coated membrane by ADH93 and ADH92. Cell invasion was allowed for 16hours with simultaneous peptide treatment. Sucrose served as the control treatment. The mean number and standard deviation of cells that invaded the matrigel membrane were calculated from 3 or more identically treated wells.

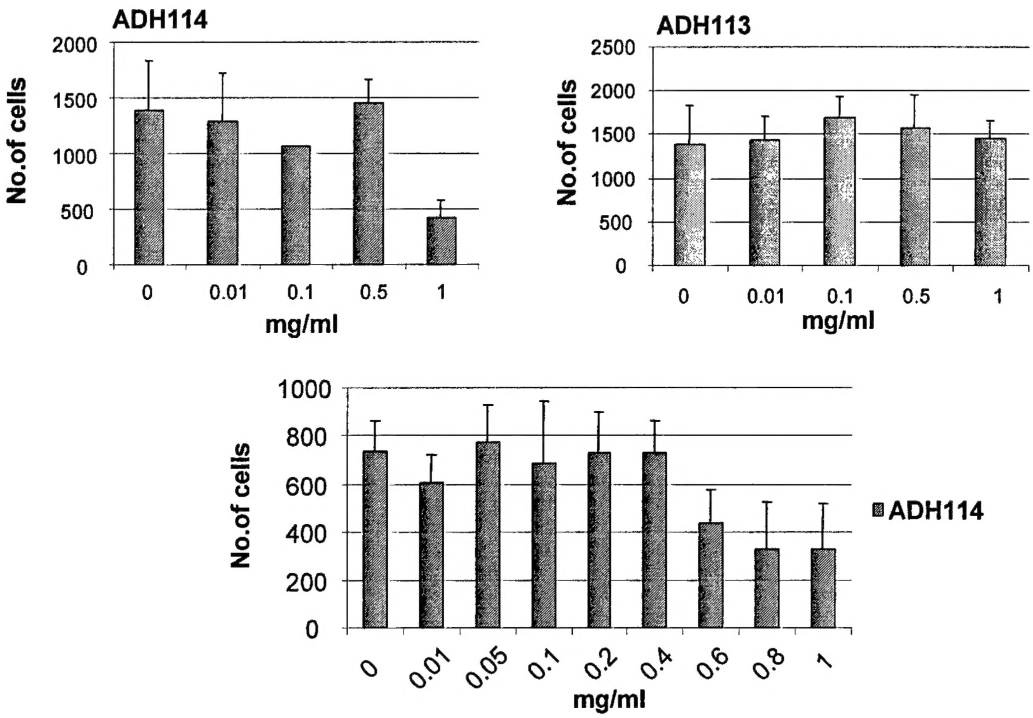

Fig. 3: Inhibition of MDA-MB-231 cell invasion through matrigel-coated membrane by ADH114 and ADH113. Cell invasion was allowed for 16 hours with simultaneous peptide treatment. No control treatment was required. The mean number and standard deviation of cells that invaded the matrigel membrane were calculated from 3 or more identically treated wells.

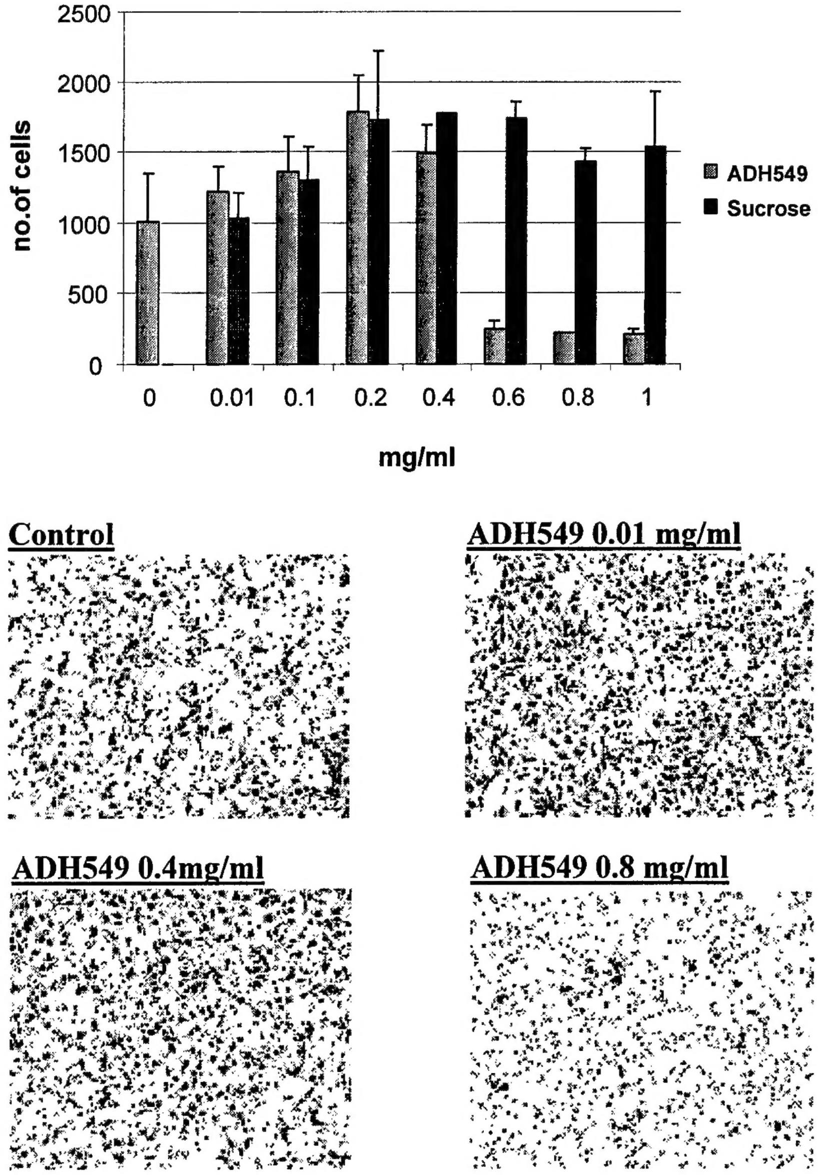

Fig 4: Inhibition of MDA-MB-231 cell invasion through a matrigel-coated membrane by ADH549. Cell invasion was allowed for 16 hours with simultaneous peptide treatment. Sucrose served as control treatment. The mean number and standard deviation of cells that invaded the matrigel membrane were calculated from 3 or more identically treated wells. Images of cell invasion were taken on Vanox Olympus microscope at x2.5 magnification.

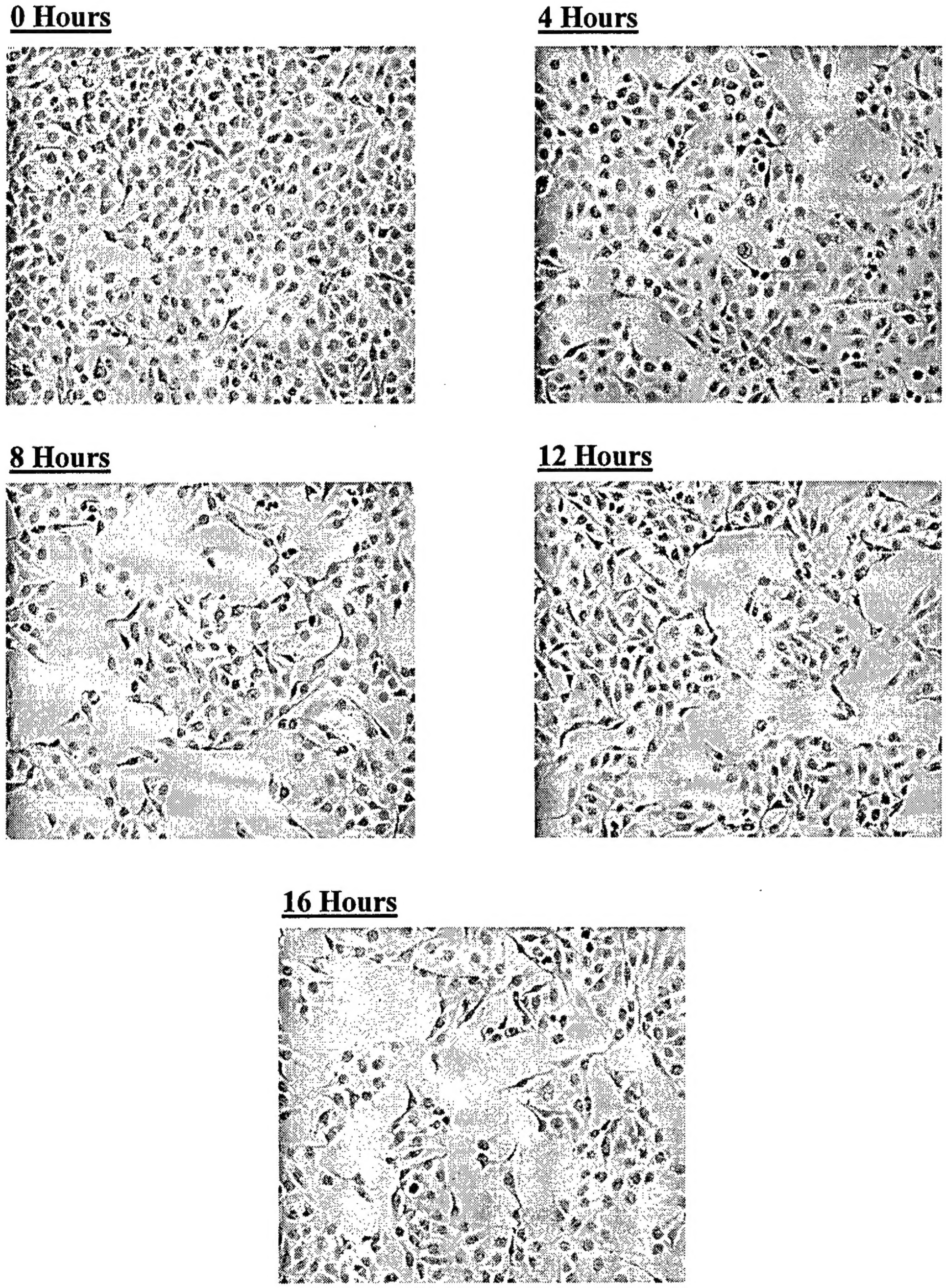

Fig. 5: Effect of ADH549 peptide on MDA-MB-231 cell morphology. Serum-starved cells were seeded on coverslips and incubated with 1mg/ml peptide in serum-free conditions for 16 hours. Samples were taken at intervals and fixed in DiffQuik fixative for 5 minutes. After washing 3 times with 1X PBS coverslips were mounted on microscopy slides and analysed at x10 magnification using a Nikon E600 fluorescent microscope.

[CANCER RESEARCH 62, 6688–6697, November 15, 2002]

An Alternatively Spliced Cadherin-11 Enhances Human Breast Cancer Cell Invasion[1]

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ABSTRACT

Although reduced levels of the epithelial cell adhesion molecule E-cadherin are often associated with poorly differentiated breast cancers, recent studies show that expression of other cadherins such as N-cadherin, P-cadherin, and the mesenchymal cadherin-11 is actually elevated in invasive breast cancers and cell lines. Cadherin-11 is unique among cadherins in that it exists as two alternatively spliced forms that are expressed together in the same cell. We now show that expression of wild-type cadherin-11, with or without coexpression of the COOH-terminal truncated splice variant, promotes epithelial differentiation of the cadherin-negative SKBR3 cell line. Exogenous wild-type cadherin-11 association with and membrane recruitment of β-catenin and p120 are unaffected by coexpression of the truncated variant. Cadherin-11-expressing cells exhibit modest changes in cell proliferation and no change in anchorage-independent growth. However, coexpression of wild-type cadherin-11 and the splice variant promotes a dramatic increase in the ability of SKBR3 cells and E-cadherin-positive MCF7 cells to traverse Matrigel-coated filters. Biochemical studies indicate that the truncated variant may be secreted from the cell and/or enter a detergent-insoluble compartment. These data suggest that the presence of the cadherin-11 splice variant promotes invasion of cadherin-11-positive breast cancer cells.

INTRODUCTION

The cadherins are a superfamily of transmembrane glycoproteins that mediate cell to cell adhesion in a variety of tissues (reviewed in Refs. 1–3). Members of the classic cadherin subfamily possess five EC domains and a conserved cytoplasmic region. These molecules are responsible for formation of the adherens junction in most cell types. Upon calcium ion binding by the extracellular region, the EC domains change conformation, allowing lateral dimerization and subsequent cross-association between dimers on adjacent cells, generally in a homotypic fashion (*i.e.,* E-cadherin to E-cadherin; Ref. 4). The cytoplasmic tail interacts directly or indirectly with "linker proteins," including β-catenin, plakoglobin or p120, α-catenin, α-actinin, and vinculin. These proteins are thought to couple cadherins to the actin cytoskeleton to strengthen the adhesive force of the entire junctional complex (5).

The best-characterized classic cadherin is E-cadherin. Differential expression of E-cadherin has been implicated in several aspects of development, including cell sorting during gastrulation and tissue morphogenesis as well as the establishment of differentiated cell identity [for example, in the intestinal lumen (6, 7)]. In addition, E-cadherin has been studied extensively with respect to its role as a putative tumor suppressor gene. Decreased E-cadherin expression has been correlated with metastasis and decreased survival in several different cancers, including breast carcinoma (8). However, E-cadherin loss is not an absolute predictor of tumor invasion or metastasis. Interestingly, increased expression of other cadherins, such as N-cadherin and P-cadherin, may also be associated with development or progression of breast carcinoma (9, 10).

Cadherin-11, or OB-cadherin, was originally identified in mouse osteoblasts (11); it was later found to be expressed in a variety of normal tissues of mesodermal origin, including areas of the kidney and brain (12). Although a type II cadherin (lacking a conserved HAV sequence), cadherin-11 is otherwise similar in structure to the type I cadherins N- and P-cadherin. In addition, the genomic structure of cadherin-11 exhibits a unique mRNA splice site, allowing for two forms of the protein to be expressed: cadherin-11; and a COOH terminus-truncated variant (Fig. 1*A*; Ref. 11). The variant has an extracellular domain identical to that of cadherin-11, but a frameshift event adds a unique cytoplasmic region with no homology to the cytoplasmic domain of any known cadherin (13). The function of the variant protein is unknown, but its sequence shows slight similarity to the src family of tyrosine kinases. In all situations thus far examined, cadherin-11 and its variant form are expressed coincidentally (14, 15).

Cadherin-11 mRNA is expressed in several types of cancer cells, including colon, gastric, renal cell, and breast cancer and osteosarcoma (14–18). In many instances, cadherin-11 expression has been associated with more aggressive, dedifferentiated cancers, such as the signet ring cell subtype of gastric carcinoma (18). In addition, we have previously shown cadherin-11 to be differentially expressed in more aggressive breast cancer cell lines (14).

However, no studies have examined the function of cadherin-11 in carcinoma cells. In the present study we find that, in contrast to cadherin-11, which localizes to the cell membrane (14), the truncated variant is found in a detergent-insoluble compartment. In addition, cadherin-11, in the presence or absence of the truncated variant, can mediate the formation of a functional adherens junction complex, recruiting β-catenin, α-catenin, and p120^{ctn} to the membrane. Finally, although expression of cadherin-11 and variant does not dramatically alter anchorage-independent growth or cellular proliferation rates, it does cause significant changes in the invasive capacity of both cell types.

MATERIALS AND METHODS

Production of Fusion Protein Vectors. Portions of cadherin-11 cDNA were subcloned into the pGEX-2TK fusion protein expression vector (Amersham Pharmacia Biotech) as follows. Using RT-PCR,[3] intracellular (WTIC) domains of wild-type cadherin-11 were amplified from total RNA derived from the MRC5 human embryonic lung cell fibroblast cell line (14). Oligonucleotide primers (For-5′-GCGGCGG*GATCCGCTGGGGAAGAAGACACAGAAG-3′; Rev-5′-CCGCCGG*AATTCTCTTGAGAACGCCAGACACAG-3′) were designed to incorporate *Bam*HI and *Eco*RI restriction enzyme digest sites into the 5′ and 3′ ends, respectively, of the resulting cDNA fragments. RT-PCR reactions were performed with the following parameters:

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[3] The abbreviations used are: RT-PCR, reverse transcription-PCR; FBS, fetal bovine serum; CAT, chloramphenicol transferase; PBS, phosphate buffered saline; LDS, lithium dodecyl sulfate; TCF, T-cell enhancer binding factor.

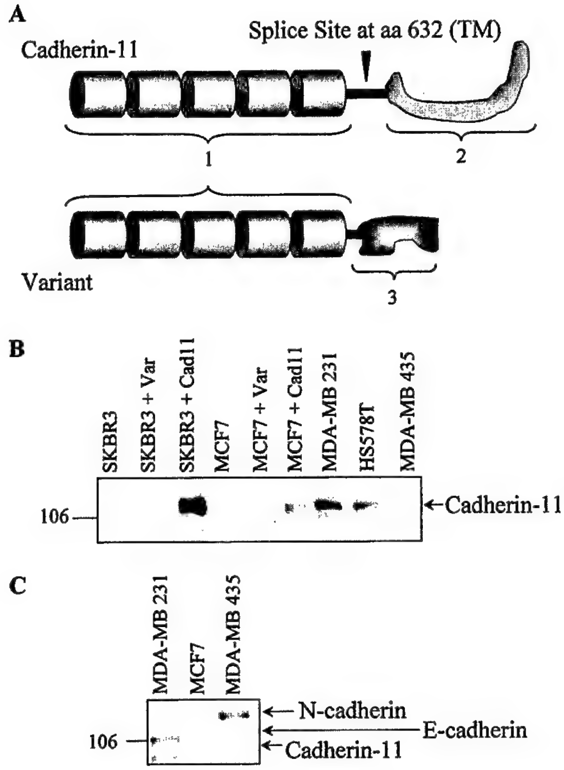

Fig. 1. *A*, the genomic sequence of cadherin-11 is depicted at the junction between exons 13 and 14, which corresponds to amino acid 632 in the transmembrane region of wild-type cadherin-11. Cadherin-11 is produced when the intron between exons 13 and 14 is removed, and the subsequent mRNA is translated. The variant protein arises when a portion of the intervening intronic sequence is alternatively spliced between exons 13 and 14 and subsequently translated. The splice event produces a frameshift and early truncation of the protein due to a stop codon 179 bp after the splice site. The resulting variant protein is therefore identical to wild-type cadherin-11 in its extracellular region and the 5′ transmembrane region (*1*). However, 3′ to the spice site, the variant (*3*) exhibits no homology to the intracellular domain of cadherin-11 (*2*; adapted from Ref. 13). *B*, NP40-soluble lysates from different breast cancer cell lines expressing endogenous or exogenous cadherins were evaluated by Western analysis to determine the specificity of monoclonal 5B2H5 antibody. SKBR3 parental cells express no known cadherins, MCF7 cells express only E-cadherin, MDA-MB-231 cells express cadherin-11, HS578T cells express N-cadherin and cadherin-11, and MDA-MB-435 cells express N-cadherin alone. In addition, SKBR3 and MCF7 cells were transiently transfected with cDNA encoding cadherin-11 or variant. A distinct M_r 120,000 band is recognized in those cells expressing cadherin-11. Note that the antibody does not recognize the variant form or cross-react with N-cadherin or E-cadherin, the two cadherins most closely related to cadherin-11. *C*, the polyclonal antibody WTID1 was characterized by analyzing lysates from MDA-MB-231 cells (cadherin-11), MCF7 cells (E-cadherin), and MDA-MB-435 cells (N-cadherin) and found to recognize all three cadherins.

reverse transcription from 1.0 μg of total RNA using mouse mammary tumor virus reverse transcriptase (Life Technologies, Inc.) for 1 h at 37°C with 0.2 μM reverse primer was followed by the addition of 0.2 μM forward primer and 30 PCR cycles (94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min). After isolation of appropriate PCR products by gel electrophoresis, the cDNA fragments were digested with *Bam*HI and *Eco*RI, gel-purified, and ligated overnight into a *Bam*HI/*Eco*RI-cut PGEX-2TK vector using T4 DNA ligase (Life Technologies, Inc.). Clones were isolated, digested to ensure correct orientation of the insert, and subsequently sequenced to verify that no mutations occurred during amplification.

Production and Isolation of Polyclonal and Monoclonal Antibodies. pGEX-2TK-WTIC was transformed into DH5α bacterial cells, and glutathione *S*-transferase fusion proteins were made and purified using standard procedures. Two rabbits were immunized and given four injections 1 week apart and then a fifth injection 2 weeks later. Rabbits were bled 10 days after the fifth booster injection, bled once more 10 days later, and then given another injection 4 days later. Serum was tested by Western blot to identify high-titer animals. After the 10th immunization, rabbits were bled by collecting 25 ml and bleeding three times, 2 days apart, and sacrificed. For purification, each two bleeds (25 ml each) following a single booster were pooled. An affinity column was made by conjugating the same peptide used for immunization to aminohexane gel by sulfo-SMCC. Serum was incubated with the gel, and the column was washed extensively. Purified antibodies were first eluted with KSCN and then with glycine. The two fractions of antibodies were dialyzed, concentrated if necessary, and then tested by immunoblot and/or immunoprecipitation and/or immunocytochemistry.

For production of monoclonal antibodies, Balb/C mice (8 weeks old) were immunized i.p. with recombinant fusion proteins mixed with complete Freund's adjuvant and then boosted several times with the same antigen mixed with incomplete Freund's adjuvant. Blood samples were collected and tested by ELISA and Western blot. Mouse splenocytes from the best responders were fused with mouse myeloma cells (P3 × 63Ag8.653) using polyethylene glycol. All hybridoma supernatant samples from the 96-well culture plates were screened by ELISA, using plates coated with the appropriate fusion protein. After expansion into 24-well plates, the positive clones were further examined by Western blot. All single-cell clones (subclones) were examined by ELISA and Western blot to confirm their production of the antibody of interest. Final subclones were isotyped by Mouse MonoAb ID Kit (catalogue number 90-6550; Zymed). Ascites from clone 5B2H5 were produced in Balb/C mice and purified using a protein A column.

Characterization of Antibodies. Characterization of antibodies was performed by Western blot analysis of several cell lines that expressed varying combinations of exogenous or endogenous cadherin-11 and variant, E-cadherin, and N-cadherin. Briefly, SKBR3 cells (which express no known cadherins) and MCF7 cells (which express E-cadherin) were transiently transfected with cadherin-11 or variant cDNA. These cells, as well as parental SKBR3 and MCF7 cells, MDA-MB-231 cells (which express cadherin-11 and variant), HS578T cells (which express cadherin-11, variant, and N-cadherin), and MDA-MB-435 cells (which express N-cadherin but not cadherin-11), were lysed in NP40 lysis buffer [1% NP40, 150 mM NaCl, 50 mM Tris (pH 8.0), 1 mM sodium vanadate, 50 mM NaF, and protease inhibitors (Boehringer Mannheim)]. The soluble fractions were isolated by centrifugation at 12,000 × *g* for 10 min at 4°C, diluted in sample buffer, and run under denaturing conditions on 4–12% Tris-glycine gels (Novex Corp.). After transfer to nitrocellulose and blocking for 1 h at room temperature in 5% nonfat milk in phosphate buffered saline (2% Tween), membranes were incubated with each antibody at varying concentrations, rinsed, and incubated with appropriate secondary antibody conjugated to peroxidase (Jackson ImmunoResearch). Blots were visualized using enhanced chemiluminescence (Amersham). All cadherin-11 antibodies were also used in immunocytochemistry, immunoprecipitation, and immunohistochemistry assays to further determine specificity as well as efficacy in these techniques.

Cell Culture and Production of Stable Transfectants. SKBR3 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured at 37°C, 5% CO_2 in DMEM (Life Technologies, Inc.) plus 10% FBS (Biofluids). Cells were transfected with pCXN2-cadherin-11 and/or pCXN2-variant or with pCDNA3-CAT (as a control), and equal amounts of the puromycin resistance plasmid pHA262pur (a gift from Dr. H. te Riele, Netherlands Cancer Institute) using the calcium phosphate method. Resistant pools were subsequently selected in 1 μg/ml puromycin for 4–6 weeks, further enriched by differential trypsinization, and characterized by Western blot, RT-PCR, and/or immunocytochemistry.

MCF7 cells were obtained from American Type Culture Collection and cultured at 37°C, 5% CO_2 in DMEM (Life Technologies, Inc.) plus 5% FBS (Biofluids). Cells were transfected with pCXN2-cadherin-11 and/or pCXN2-variant or with pCDNA3-CAT (as a control) using LipofectAMINE Plus (Life Technologies, Inc.) and selected in 0.5 mg/ml G418 for 4–6 weeks. Clonal populations of cells were obtained by plating to a limiting dilution in 0.5 mg/ml G418; subsequent clonal populations were screened by immunocytochemistry and Western blot analysis.

Antibodies. The following primary antibodies were used for immunocytochemistry, immunoprecipitation, and immunoblotting: (*a*) monoclonal anti-cadherin-11 clone 5B2H5 and polyclonal anti-cadherin-11 (WTID; Zymed), both of which were raised against the intracellular domain of cadherin-11 and hence recognize the wild-type cadherin-11 alone; (*b*) monoclonal anti-cadherin-11 (a gift from M. J. Bussemakers; 228) and monoclonal anti-cadherin-11 113H (a gift from the ICOS Corp.), both of which recognize the extracellular domain of cadherin-11 and variant; (*c*) monoclonal anti-β-catenin (Transduction Laboratories); (*d*) polyclonal anti-β-catenin SHB7 (a gift from

D. Rimm); (*e*) monoclonal anti-p120^{ctn} (Transduction Laboratories); and (*f*) monoclonal anti-p120^{ctn} clone 12F4 (a gift from A. B. Reynolds).

Immunocytochemistry and Microscopy. Cells were plated on sterile 18-mm glass coverslips and allowed to adhere for at least 48 h before fixation in 2% paraformaldehyde (20 min), with subsequent permeabilization in 0.2% Triton X-100-PBS (5 min). Cells were blocked for 1 h at room temperature in 3% ovalbumin-PBS and then incubated with the appropriate antibody diluted in 6% normal goat serum-PBS for 1 h at room temperature. After three 3-min washes in PBS, cells were incubated with the appropriate secondary antibodies conjugated to either Texas Red (Jackson ImmunoResearch) or FITC (Kirkegaard & Perry) for 1 h at room temperature in the dark. All secondary antibodies were diluted 1:200 in 6% normal goat serum-PBS. For double staining, polyclonal primary and antirabbit secondary antibodies were applied first, followed by monoclonal primary and antimouse secondary antibodies. After the final three 3-min washes in PBS, coverslips were mounted on slides with Vectashield. All fluorescence and Nomarski interference contrast images were digitally captured using an Olympus fluoview confocal microscope.

Immunoblotting and Immunoprecipitation. Cells were solubilized in ice-cold 1% NP40 buffer solution [1% NP40, 1250 mM NaCl, and 50 mM Tris (pH 8.0)] containing 1 mM sodium vanadate, 50 mM NaF, and complete protease inhibitors (Boehringer Mannheim). Lysates were centrifuged at 14,000 rpm for 15 min at 4°C to remove the NP40-insoluble material. After the addition of 2× sample buffer [4% SDS, 120 mM Tris (pH 6.8), and 20% glycerol] to the NP40-soluble fraction and 1× sample buffer to the insoluble pellet, samples were boiled, and Bio-Rad protein assays were performed to determine total protein content. After the addition of reducing agent, the samples were again boiled, and equal total protein was loaded on 3–8% NuPage Tris-acetate gels (Invitrogen), unless otherwise indicated. Proteins were blotted to nitrocellulose (Schleicher & Schuell) and blocked for 1 h at room temperature or overnight at 4°C in 5% milk-PBST. After incubation with appropriate primary and secondary antibodies (each for 1 h at room temperature), blots were treated with enhanced chemiluminescence reagent (Amersham) and exposed to film. Blots were sometimes stripped [62.5 mM Tris (pH 7.5), 2% SDS, and 1.7% B-mercaptoethanol for 30 min at 50°C], reblocked, and reprobed with new primary and secondary antibody. Alternatively, cells were lysed in ice-cold 1× LDS sample buffer (Invitrogen), scraped, passed through a 27-gauge needle, and boiled in the presence of a reducing agent for 30 min before gel electrophoresis and immunoblotting as described.

For immunoprecipitation, lysates were obtained as described above. Lysates were first precleared with 50 μl of protein G-Sepharose beads (Zymed) alone for 1 h at 4°C. The beads were centrifuged and removed, and appropriate precipitating antibodies were added to lysates for 1 h with rocking at 4°C; 50 μl of new beads were then added with rocking for an additional 2 h at 4°C. After three washes with ice-cold lysis buffer, sample buffer and reducing agent were added to precipitated proteins and beads and boiled for 10 min; samples were subsequently analyzed by immunoblotting as described above.

Reporter Assays. Twelve-well plates were seeded at 10^5 cells/well 24–48 h before transfection. Cells were transfected with TOPFLASH reporter (indicates lymphocyte enhanced binding factor reporter activity), pCXN2-cadherin-11 and pCXN2-variant or pCDNA3-CAT (control), and the thymidine kinase *Renilla* luciferase plasmid (Promega) to control for variations in transfection efficiency. After lysis, luciferase and *Renilla* activities were read on a standard luminometer using the Dual-Reagent Luciferase Assay Kit (Promega). Luciferase values were normalized to *Renilla* values and plotted using Sigma Plot. Each experiment was performed in triplicate at least three independent times, with error bars representing SD.

Proliferation Assays. WST-1 assays (Boehringer Mannheim) were performed as indicated by the manufacturer. Briefly, 1000–3000 cells of each population were plated in triplicate in 96-well plates on day 1. WST-1 readings were taken on alternate days beginning with day 0 on an optical densitometer. For analysis (Sigma Plot), data for each population were plotted relative to the mean day 0 value to account for variance in plating efficiency, with error bars representing SD. All experiments were performed independently at least three times.

Soft Agar Assays. Soft agar assays were performed as described previously (19). Briefly, cells were plated in 6-well plates at 5×10^5 cells/well in a 0.3% agar suspension (SeaKem) on a 0.6% agar cushion, with 1 ml of DMEM + 5% FBS covering the cells. The cells were incubated at 37°C in 5% CO_2, and media were carefully changed every 3–4 days. After 2 or 3 weeks, colonies greater than 140 μm in diameter were scored by an Omnicon 3600 Colony Counter; data were subsequently analyzed and graphed on Sigma Plot. All experiments were performed in triplicate at least three independent times; error bars represent SD.

***In Vitro* Invasion Assays.** Invasion assays were performed as described previously (9, 20). Standard Boyden chambers were prepared by placing NIH3T3-conditioned media (24 h, DMEM +50 μg/ml ascorbic acid) in the bottom well of the chamber as a chemoattractant. After coating a 12-μm pore size polycarbonate filter (Poretics, Inc.) with 10 μg of Matrigel, 3×10^5 cells in DMEM with 0.1% BSA were placed in the upper chamber and incubated for 16 h at 37°C. Membranes were then removed, cells were fixed in 25% methanol with 0.5% crystal violet, and the remaining cells were wiped from the upper surface of the membrane with a damp cotton swab. Quantification of cells on the bottom of the membrane was performed by counting the number of cells/field in 5 random fields/membrane; the fields were then averaged. Bars represent the mean of each population over three membranes, with error bars representing SD. Each experiment was performed at least three independent times.

RESULTS

Cadherin-11 Antibody Production and Characterization. To characterize the monoclonal and polyclonal antibodies raised against cadherin-11, we first determined their specificity by Western analysis. Lysates from MDA-MB-231 cells were evaluated because these cells express both forms of cadherin-11 (14). In addition, we examined lysates from MCF7, HS578T, and MDA-MB-435 cells, which express E-cadherin, N-cadherin and cadherin-11, and N-cadherin, respectively (14, 20). Cadherin-11 is relatively homologous to E- and N-cadherin; anti-cadherin-11 antibodies, especially those raised against the intracellular domain, were therefore most likely to cross-react with these proteins. Finally, to determine whether antibodies could recognize exogenously expressed cadherin-11, we evaluated lysates from both SKBR3 cells and MCF7 cells transiently transfected with cDNA encoding the wild-type cadherin-11 or variant protein.

One monoclonal antibody (5B2H5) to the intracellular domain of cadherin-11 was reactive by Western blot, indicated by the M_r ~115,000 species (Fig. 1*B*). 5B2H5 did not react with the variant form or with any proteins in the MCF7 or MDA-MB-435 lanes (E-cadherin and N-cadherin, respectively), indicating that it was specific to the wild-type form of cadherin-11. In addition, the only band seen in the HS578T lane is M_r 115,000, indicating that it is cadherin-11; N-cadherin runs at approximately M_r 140,000. The 5B2H5 clone was found to be useful for immunocytochemistry, immunoprecipitation, and immunohistochemistry and is available commercially as Zymed 32-1700 (clone 5B2H5).

One polyclonal antibody raised against the intracellular domain of wild-type cadherin-11 was also analyzed and found to recognize not only cadherin-11 but also E-cadherin and N-cadherin (Fig. 1*C*). This antibody (pWTID1) is also useful for immunocytochemistry; its utility in immunoprecipitation or immunohistochemistry was not investigated due to its cross-reactivity with E- and N-cadherin. This pan-cadherin antibody is available commercially as Zymed 71-7600 (cadherin-11 WTID1). We were not successful in generating monoclonal or polyclonal antibodies specific to the variant intracellular domain.

Characterization of Stable Transfectants Expressing Cadherin-11. Both SKBR3 and MCF7 breast cancer cells are relatively well differentiated and moderately invasive in *in vitro* invasion assays. Because SKBR3 cells have a homozygous deletion of the *E-cadherin* gene (21) and do not express other cadherins, they represent a suitable system for determining whether or not cadherin-11 could act as a functional adherens junction molecule in addition to whether or not it could induce a phenotypic change. The MCF7 cell line, which ex-

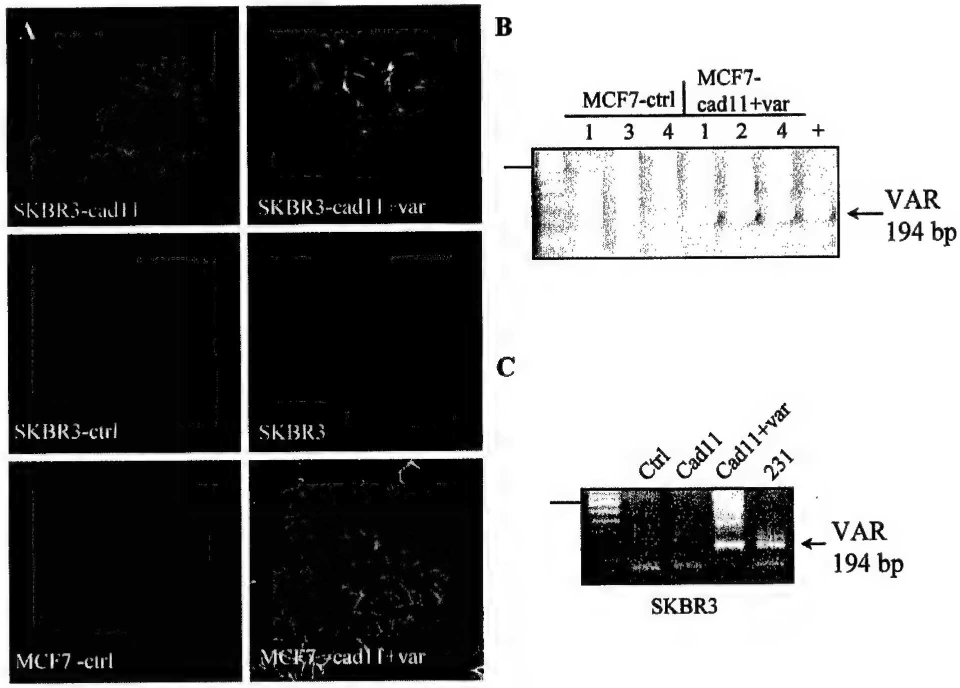

Fig. 2. *A*, SKBR3 cells were cotransfected with cadherin-11 without or with variant (SKBR3-cad11 or SKBR3-cad11+var, respectively) or pcDNA-CAT as a control (SKBR3-ctrl) and a puromycin resistance plasmid; selection resulted in pooled populations expressing the protein(s) of interest. MCF7 cells were transfected with cadherin-11 and variant (MCF7-cad11+var) or pCDNA-CAT (MCF7-ctrl) and selected with G418 before selecting clonal populations. To verify protein expression, cells were stained for cadherin-11. *B*, RT-PCR was used to verify production of variant mRNA. Note that control cells do not express the 194-bp PCR product. MDA-MB-231 cells (+), which express cadherin-11 and variant mRNA (14), were used as a positive control. *C*, RT-PCR was performed to ensure that the stably transfected SKBR3 cells expressed cadherin-11 variant mRNA.

presses E-cadherin alone, is an appropriate model to determine whether exogenous cadherin-11 expression interferes with E-cadherin function as well as induces a more proliferative or invasive phenotype in the presence of E-cadherin.

Cells were cotransfected with vectors containing the full-length cDNA coding for human wild-type cadherin-11 and/or human variant cadherin-11 (pCXN2-cadherin-11 and pCXN2-variant) or empty vector containing the CAT cDNA for control, with or without a second puromycin resistance plasmid (pHA262pur). Pooled stable populations expressing cadherin-11, both cadherin-11 and the variant, or CAT were obtained either by selection with G418 or puromycin. These cell populations will be referred hereafter to as SKBR3-cad11, SKBR3-cad11+var, and SKBR3-ctrl, respectively.

MCF7 cells were transfected with cadherin-11 and variant or CAT as described above; selection with G418 resulted in pooled populations 40–90% positive for cadherin-11. To obtain populations in which 100% of cells expressed the appropriate protein(s), we selected clones by limiting dilution in G418 with subsequent screening by immunocytochemistry (Fig. 2*A*). Further characterization by RT-PCR analysis revealed production of the cadherin-11 variant in all clones (Fig. 2*B*). These cell populations will be referred to hereafter as MCF7-cad11+var and MCF7-ctrl, respectively.

Expression of Cadherin-11 Results in Morphological Changes in SKBR3 Cells. SKBR3 cells were cotransfected with the puromycin resistance plasmid pHA262pur and cadherin-11, cadherin-11 and variant, or CAT cDNA. Selection with puromycin produced populations of SKBR3 cells transfected with cadherin-11 and variant that were 90–100% positive without the need for further sorting (Fig. 2*A*). Because we had no antibody specific to the variant protein, cells were further characterized by RT-PCR analysis (Fig. 2*C*). Using primers specific for the variant mRNA species, it was clear that only SKBR3 cells transfected with the variant cDNA expressed the variant mRNA (MDA-MB-231 cells were used as a positive control). SKBR3 cells transfected with only wild-type cadherin-11 and selected in puromycin were approximately 40% positive for cadherin-11; this was further enriched to ~100% by differential trypsinization because SKBR3 cells expressing cadherin-11 adhered to plastic much more efficiently than control cells. Neither CAT-transfected nor parental SKBR3 cells express cadherin-11.

In SKBR3 cells, the expression of cadherin-11 alone or with the variant produced a profound change in morphology (Fig. 3*A*). Parental SKBR3 cells (which express no known cadherins) are weakly attached to one another, resulting in loose aggregates of cells that detach easily from one another and from tissue culture-treated plastic, especially when grown to high density. Cells expressing cadherin-11 or cadherin-11 and the variant formed closely adherent islands of cells; some of these islands grew quite large, incorporating hundreds of cells (Fig. 3*A*). These foci were significantly more difficult to dislodge from plastic with trypsinization than parental or control cells, suggesting an increased ability to adhere to one another and to the extracellular substrate. No differences were observed in the morphology or trypsinization properties of cells expressing wild-type cadherin-11 compared with those expressing both cadherin-11 and the variant.

MCF7 clones expressing cadherin-11 and the variant did not exhibit any noticeable changes in morphology when compared with control cells (data not shown). This is probably because MCF7 cells already express E-cadherin, which confers strong cell-cell adhesion; it is unlikely that cadherin-11 expression would further increase this adhesion (20).

Cadherin-11 Expression Results in the Assembly of Adherens Junction Components. As discussed earlier, cadherin-11 is generally expressed in mesenchymal cells or cells with a mesenchymal phenotype, such as invasive cancer cells. These types of cells do not usually form stable adherens junctions. However, the morphological changes that accompany the expression of cadherin-11 in SKBR3 cells suggest that this cadherin, like E-cadherin, can promote homotypic cell adhesion in addition to mediating the formation of adherens junctions. We previously showed that β-catenin interacts with cadherin-11 in cells expressing both proteins endogenously (14). In contrast, Thoreson *et al.* (22) suggested that another adherens junction component and member of the catenin family, $p120^{ctn}$, does not interact with cadherin-11. They demonstrated that $p120^{ctn}$ does not localize to the membrane in MDA-MB-231 cells, which express only one known cadherin, cadherin-11, in the membrane at sites of cell-cell

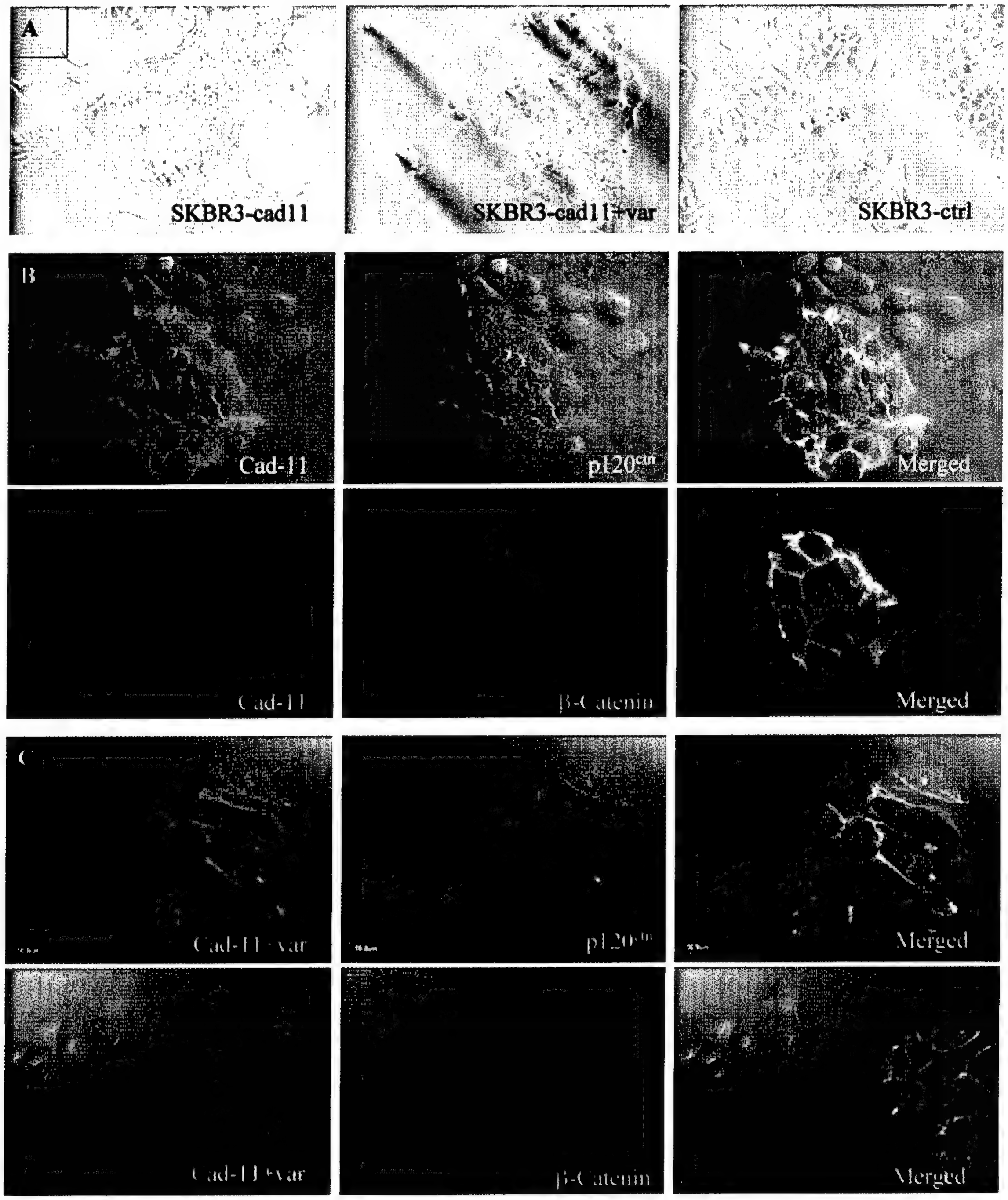

Fig. 3. *A*, phase-contrast microscopy of SKBR3 cells stably expressing wild-type cadherin-11 without or with variant compared with control cells. *B*, SKBR3 cells expressing cadherin-11 were stained for β-catenin, p120, and cadherin-11. *C*, double-labeling for cadherin-11 and either β-catenin or p120^{ctn} was performed in SKBR3 cells stably expressing cadherin-11 and variant proteins.

contact (22). We independently confirmed these results (data not shown). The localization of p120^{ctn} in the cytoplasm of MDA-MB-231 cells, instead of at the membrane with cadherin-11, suggests that these molecules might not interact with one another. We therefore tested the ability of cadherin-11 to recruit members of the adherens junction complex to the membrane of SKBR3 cells.

Cadherin-11-expressing SKBR3 cells were stained for cadherin-11, p120^{ctn}, and β-catenin and visualized by confocal microscopy (Fig. 3*B*). These experiments were carried out on puromycin-selected pooled stable expressers before final enrichment (see "Materials and Methods"). Consequently, not all cells express cadherin-11, allowing the negative cells to act as internal controls. First, we analyzed the expression pattern of cadherin-11. It was noted that SKBR3-cad11 cells localize the protein to the membrane and only do so at sites of cell-cell contact. Note that there is no cadherin-11 protein at the edges of cells unless they contact other cells. Next, we examined the expression of p120^{ctn} in these cells. We found a marked relocation of p120^{ctn} to the membrane only in those cells concomitantly expressing cadherin-11. Merged image analysis indicates that cadherin-11 and p120^{ctn} colocalize, suggesting that cadherin-11 does indeed have the capability to interact with and recruit p120^{ctn}. Staining for β-catenin (Fig. 3*B*) shows that cadherin-11 also recruits this protein to the membrane. Colocalization of the two proteins is clear in the merged image. Finally, concurrent expression of the variant form of cadherin-11 did not appear to alter the localization of either catenin (Fig. 3*C*). Similar

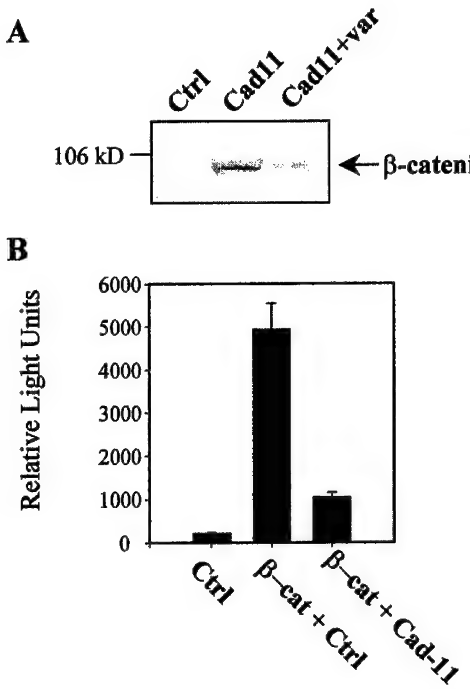

Fig. 4. *A*, NP40-soluble fractions of SKBR3-ctrl, SKBR3-cad11, and SKBR3-cad11+var cells were analyzed by Western blot for β-catenin protein. SKBR3 cells normally express very low levels of β-catenin protein (*Ctrl*). Expression of cadherin-11 (*Cad11*) or of cadherin-11 and variant (*Cad11+var*) increased β-catenin protein levels. Although β-catenin appears to be more stable in cells expressing cadherin-11 than in cells expressing both cadherin-11 and variant, this is because the SKBR3-cad11 population is more enriched for cadherin-11 than the SKBR3-cad11+var population at the time of this experiment. *B*, SKBR3 cells were transiently transfected with a β-catenin/LEF reporter construct, TOPFLASH, or with pcDNA-CAT (control). β-Catenin increased TOPFLASH activity roughly 20-fold, and cadherin-11 reduces β-catenin signaling 5-fold, indicating that cadherin-11 effectively removes a majority of β-catenin from the signaling pool, likely by stabilizing it at the membrane.

experiments were also performed in MCF7 cells. These cells already express E-cadherin; coexpression of cadherin-11 and variant did not alter the distribution of β-catenin or $p120^{ctn}$ (data not shown).

Parental SKBR3 cells normally exhibit barely detectable levels of cytoplasmic β-catenin, similar to the cadherin-11-negative cells in the margins of the image in Fig. 3, *B* and *C* (20). Our findings suggested that cadherin-11 might be stabilizing β-catenin (*i.e.,* preventing its degradation) by recruiting it to the cell membrane. To confirm this, a Western blot analysis for β-catenin protein was performed on control SKBR3 cells, cells expressing cadherin-11, and cells expression both cadherin-11 and the variant. Fig. 4*A* reveals that β-catenin protein levels are markedly elevated in cells expressing cadherin-11 compared with control cells. Additionally, it has been shown previously in other systems that expression of E-cadherin can recruit β-catenin to the membrane, thus reducing its ability to activate TCF reporters (23). Fig. 4*B* shows that expression of cadherin-11 can also reduce the ability of β-catenin to activate TCF reporters in a similar manner.

To verify that cadherin-11 exists in a complex with both β-catenin and $p120^{ctn}$, instead of simply colocalizing in the same vicinity, immunoprecipitation experiments were performed (Fig. 5). Both β-catenin and $p120^{ctn}$ were present in cadherin-11 immunoprecipitations from cadherin-11-expressing SKBR3 cells. It was also noted that the β-catenin that precipitated with cadherin-11 has a slower electrophoretic mobility than the β-catenin found in the cell lysate. Finally, reprobing of these blots determined that a third catenin, α-catenin, is present in the complex and also appears to be of a slightly higher molecular weight than the protein found in the cell lysate (Fig. 5*A*).

We also wished to determine whether the expression and activity of cadherin-11 and variant proteins might affect or be affected by the presence of E-cadherin. We therefore immunoprecipitated either cadherin-11 or E-cadherin from parental MCF7 cells and cadherin-11-expressing MCF7 cells and blotted for the same catenins described above (Fig. 5, *B* and *C*). We found that neither cadherin-11 nor E-cadherin prevented the other from associating with $p120^{ctn}$, β-catenin, or α-catenin. In addition, $p120^{ctn}$ appeared to immunoprecipitate more readily with cadherin-11 than with E-cadherin. Finally, expression of cadherin-11 does not appear to alter the steady-state levels of E-cadherin expression.

Effects of Exogenous Cadherin-11 on Cellular Proliferation. To determine whether exogenous expression of cadherin-11 might alter the cellular phenotype, we evaluated several indicators of cellular behavior, including proliferation rate, anchorage-independent growth, and invasive activity. The rate of cell proliferation was assessed by plating cells at varying densities and then monitoring their growth using the WST-1 assay, which measures cellular metabolic activity. Expression of wild-type cadherin-11 in SKBR3 cells led to a moderate change in the proliferation rate of cells (Fig. 6*A*). At the peak of the proliferation log phase, SKBR3-cad11 cells exhibited approximately 150% the growth rate of controls. In SKBR3-cad11+var cells, the growth rate was approximately 60% that of control cells. The difference in cell proliferation between the two cadherin-11-expressing cell populations at log peak was significant ($P < 0.001$). However, coexpression of cadherin-11 and variant proteins did not alter the peak of the proliferation log phase for these cells. Instead, it took them longer to reach log phase than controls (14 days *versus* 6 days). These data suggest that expression of cadherin-11 alone may confer a slight proliferative advantage to SKBR3 cells, which appears to be reversed by concomitant expression of the variant protein. It should be noted that although the observed changes in proliferation rate assessed by the WST-1 assay were repeatable and statistically significant, they were not large. For example, we did not notice in the daily management of the cells that they needed to be passaged at different times. In MCF7 cells, no consistent differences in the rate of proliferation could be observed among the various control and cadherin-11-expressing cells (data not shown).

Exogenous Cadherin-11 Does Not Affect Anchorage-independent Growth. The ability of cells to grow in soft agar is not always related to changes in proliferation. Consequently, it is possible that although cadherin-11 expression does not have a major effect on cell growth, it may affect the ability of cells to grow in an anchorage-independent manner. The effect of cadherin-11 expression on anchorage-independent growth was examined in both SKBR3 and MCF7 cells by culturing them on a cushion of soft agar for 2 weeks. We found that the ability of SKBR3 and MCF7 cells to grow in an anchorage-independent fashion was not affected by expression of cadherin-11 or by coexpression of cadherin-11 and variant proteins (Fig. 6, *B* and *C*).

Exogenous Cadherin-11 Affects Invasive Activity. We reported previously that cadherin-11 is expressed in breast cancer cell lines displaying a more aggressive and invasive phenotype. We therefore wished to determine whether cadherin-11 expression might actually have a causal role in the acquisition of invasive capacity and tested the ability of cadherin-11-expressing cells to invade a Matrigel-coated membrane in a standard Boyden chamber assay. Equal numbers of control and cadherin-11-expressing cells were trypsinized and then plated onto Matrigel-coated filters above a well filled with chemoattractant-enriched media. Cells were then incubated for 16 h to assess their ability to move through the Matrigel toward the chemoattractant on the other side of the membrane. We found that expression of cadherin-11 in SKBR3 cells led to a 5-fold reduction in invasive capacity (compared with control or parental cells) that was statistically significant ($P < 0.05$; Fig. 7). In contrast, cells coexpressing

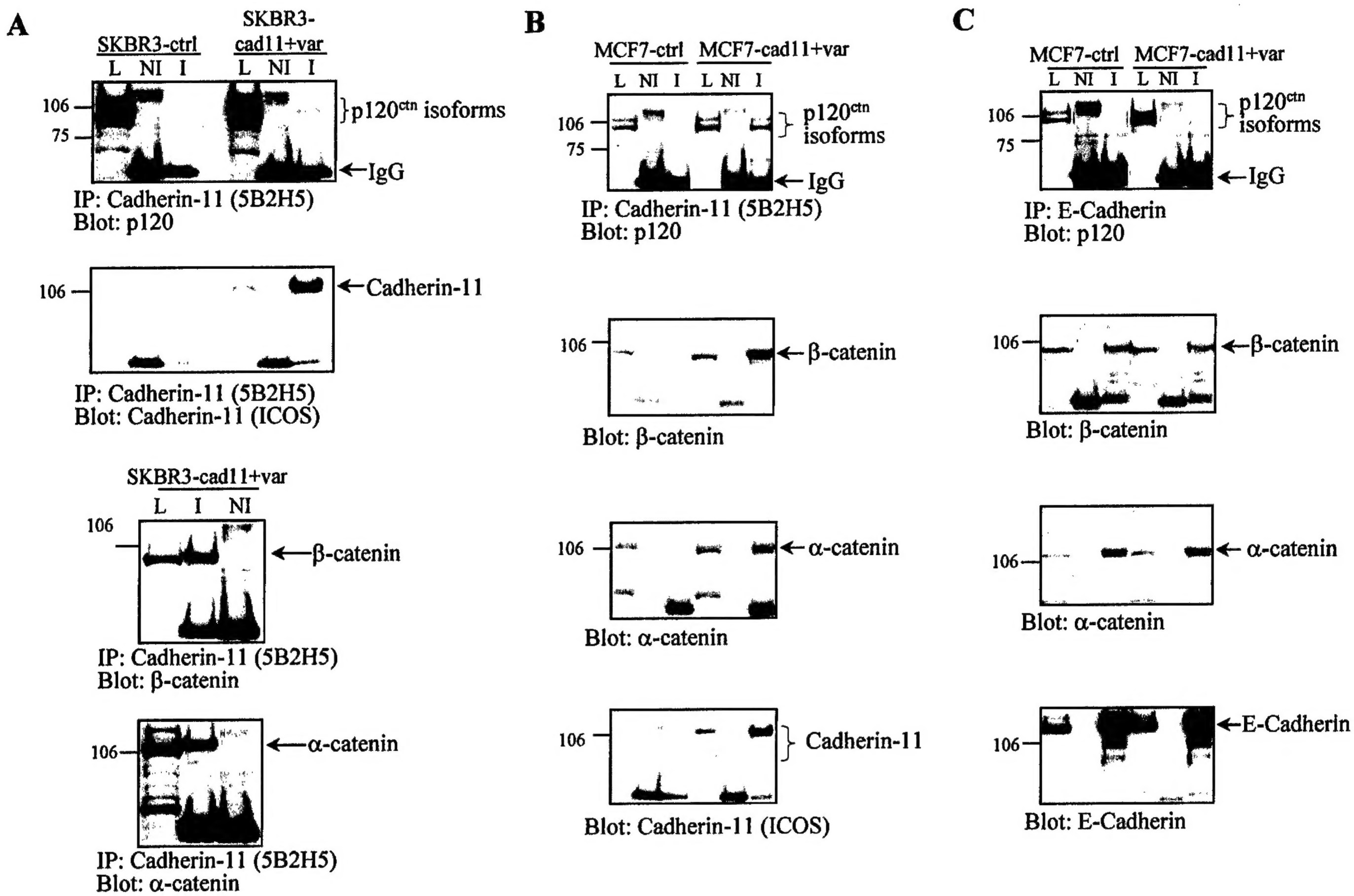

Fig. 5. *A*, immunoprecipitation of cadherin-11 was performed from either SKBR3-ctrl cells (control) or SKBR3-cad11+var cells. Blotting with an anti-p120 antibody (Transduction Laboratories) reveals that p120^{ctn} is pulled down with cadherin-11. Stripping and reprobing of the original blot prove that wild-type cadherin-11 was in fact precipitated with 5B2H5. In addition, subsequent immunoprecipitations show that both β-catenin and α-catenin can be immunoprecipitated with cadherin-11. Finally, note that both β-catenin and α-catenin appear to have shifted to higher molecular weights in the immune complexes associated with cadherin-11 as compared with the cellular pool seen in the lysate lanes. *L* refers to whole cell lysates, *I* refers to the immune complex, and *NI* refers to the nonimmune control lane. *B*, immunoprecipitations of either cadherin-11 or E-cadherin in either control cells (MCF7-ctrl) or stable expressers of cadherin-11 and variant (MCF7-cad11+var cells). Cadherin-11 coprecipitates in a complex with all three catenins. E-cadherin (Transduction Laboratories) was also immunoprecipitated and followed by blotting for the catenins. Note that there is no difference in the amount of any catenin pulled down by E-cadherin in control cells *versus* expressers, indicating that cadherin-11 does not interfere with E-cadherin association with adherens junction components.

cadherin-11 and the variant protein invaded at twice the rate of control cells ($P < 0.05$) and 10 times more than cells expressing cadherin-11 alone ($P < 0.001$). Despite these clear differences in invasive behavior, both populations of cells were morphologically indistinguishable when viewed by phase-contrast microscopy (Fig. 3*A*).

Nieman *et al.* (24) showed previously that expression of cadherin-11 in the E- and P-cadherin-expressing cell line BT20 slightly increased the invasive activity of the cells. It was not clear whether both cadherin-11 and the variant protein were expressed in these experiments, but it is possible that the effects of cadherin-11 on invasion might be different in cells that express other cadherins. To confirm that cadherin-11 may affect invasion by cells already expressing other cadherins, we repeated these experiments in MCF7 cells. Coexpression of cadherin-11 and variant increased the invasive capacity of MCF7 cells 7-fold compared with parental cells or control (Fig. 7). This increase in invasive capacity occurs despite expression of E-cadherin in these cells, indicating that cadherin-11 may in some way alter the tumor-suppressive activities of E-cadherin or that its ability to bring about an invasive phenotype is independent of E-cadherin.

Localization of the Cadherin-11 Variant. The marked effects of variant protein expression on the invasive behavior of SKBR3 and MCF7 cells prompted us to reexamine its cellular localization. Because we could not generate antibodies that specifically recognized the variant, we were forced to rely on antibodies that detected both cadherin-11 forms (*i.e.*, wild-type and variant). In earlier experiments, we found that a protein corresponding to the predicted size of the variant (M_r ~85,000 *versus* M_r 115,000 for wild-type cadherin-11) was present in cells expressing endogenous cadherin-11 (14). However, in the present study, we found that cells transfected with wild-type cadherin-11 cDNA but not variant cDNA also expressed a protein of similar molecular weight (Fig. 8*A*). Because the variant form is a product of alternative splicing of the primary RNA transcript, it cannot have been present in these cells; we hypothesize that the M_r ~85,000 protein is instead a protein degradation product of wild-type cadherin-11.

Interestingly, we found that it was difficult to detect the variant in transfected cells by either immunocytochemistry or Western blot of NP40-soluble extracts, despite abundant expression of variant mRNA. Analysis of the NP40-insoluble fraction confirmed that whereas wild-type cadherin-11 is predominantly in the NP40-soluble fraction as published previously (14), the variant is located in the NP40-insoluble, LDS-soluble fraction (Fig. 8*B*). This cellular fraction contains intracellular cytoskeletal and associated proteins as well as extracellular matrix.

However, the fact that the variant could not be detected within the cell by immunocytochemistry led us to speculate that it must instead be outside the cell. We hypothesized that the loss of two-thirds of the

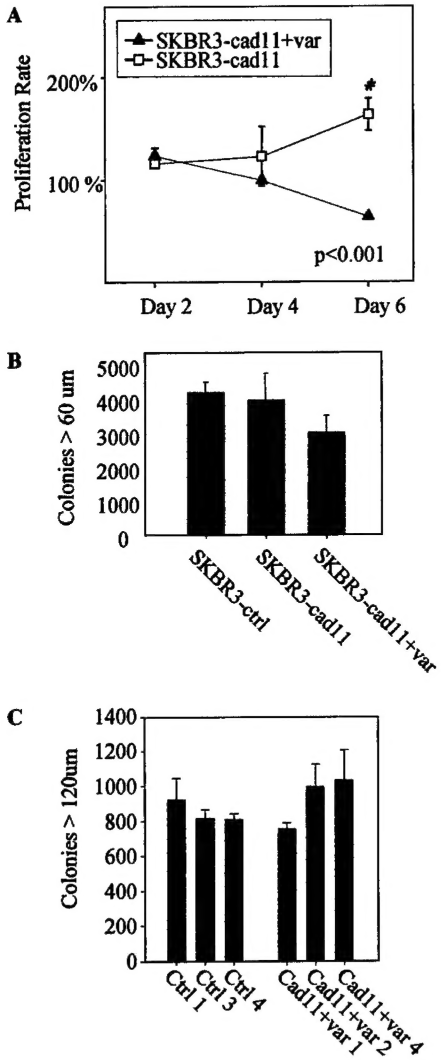

Fig. 6. *A*, proliferation rates of SKBR3 cells stably expressing CAT (SKBR3-ctrl), cadherin-11 (SKBR3-cad11), or cadherin-11 and variant (SKBR3-cad+var) were assessed using the WST-1 assay. Data were plotted relative to mean day 0 for each cell population to account for differences in plating efficiency. Additionally, both population rates were evaluated as a percentage of their respective controls to simplify comparison. *B*, anchorage-independent growth was assessed by culturing cells in soft agar. SKBR3 populations grow poorly in soft agar, regardless of cadherin-11 expression. *C*, growth was assessed as described in *B*, but in MCF7 cells. Cadherin-11 expression has little effect on anchorage-independent growth in these cells.

transmembrane domain in the variant might cause it to be secreted from the cells. To test this, SKBR3-cad11 cells were transiently transfected with variant or CAT cDNA. Twenty-four h after transfection, cells were incubated for an additional 24 h at 22°C to slow protein secretion (25) and then stained for cadherin-11. We could clearly detect the variant protein inside the variant-transfected cells by immunocytochemistry (Fig. 8*C*). Because we could not detect the variant in cells in which secretion was not blocked, we interpreted this result as suggesting that the variant form may indeed normally be secreted.

We therefore attempted to isolate variant protein from media conditioned by cells transiently transfected with variant cDNA. Twelve h after transfection, cells were placed in low-serum media (0.1%) for 72 h. Media were then collected and concentrated 40-fold in the presence of protease inhibitors. Immunoprecipitation of the variant from media was attempted using two antibodies that recognize the extracellular domain; neat media were also analyzed for variant protein by Western blot. In addition, cell lysates were examined to ensure adequacy of transfection. Despite adequate transfection, we were unable to detect variant in the conditioned media (Fig. 8*D*).

DISCUSSION

The acquisition of an invasive or metastatic phenotype by tumor cells is a complex process involving changes in a number of cellular characteristics and behaviors. Changes in cell adhesion are thought to be particularly important (26, 27). In the present study, we show that cadherin-11 with or without expression of the variant form can recruit components of the adherens junction to the membrane and result in epithelioid differentiation of SKBR3 cells. Expression of cadherin-11 and its variant has little effect on cell proliferation and anchorage-independent growth but does markedly alter the invasive ability of breast cancer cells, even in the presence of E-cadherin. Our results also suggest that the variant protein may be secreted and/or deposited into a NP40-insoluble intra- or extracellular compartment.

Cadherin-11 Recruits Components of the Adherens Junction and Promotes Epithelial Differentiation of SKBR3 Cells. Cadherin-11 was originally identified in osteoblasts and subsequently found in many cells of mesodermal origin as well as in certain invasive tumors (11, 12). Because most of these cadherin-11-expressing cells do not form stable adherens junctions, it is quite surprising that cadherin-11 expression in SKBR3 breast cancer cells results in the recruitment of adherens junction proteins to the membrane. We found that cadherin-11 brings adherens junction components such as β-catenin, α-catenin, and $p120^{ctn}$ to the membrane in both cells lacking endogenous cadherins and cells possessing endogenous cadherins (SKBR3 cells and MCF7 cells, respectively). Additionally, this recruitment is not affected by concomitant expression of the variant protein. Finally, β-catenin protein levels are themselves stabilized by cadherin-11 expression in SKBR3 cells, which

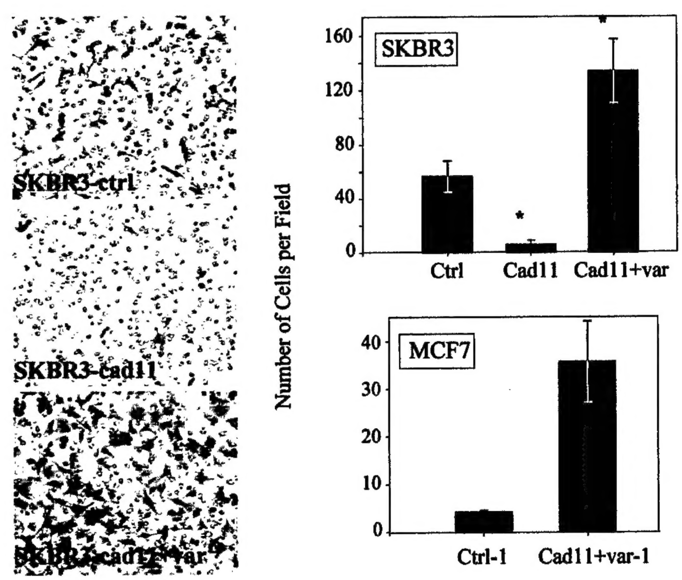

Fig. 7. Invasive capacity of SKBR3 populations was assessed using a standard Boyden chamber assay, in which cells invade a Matrigel-covered porous membrane to migrate toward chemoattractant-enriched media. Representative membrane fields from each SKBR3 population are depicted on the *left*, and the average number of cells that invaded in a 16-h period is depicted on the *right* for both SKBR3 and MCF7 cells. Expression of cadherin-11 alone (SKBR3-cad11) inhibits the invasive activity of SKBR3 cells ($P < 0.05$, SKBR3-cad11 *versus* SKBR3-ctrl). Cells coexpressing cadherin-11 and variant (SKBR3-cad11+var) invaded twice as fast as control cells ($P < 0.05$, SKBR3-cad11+var *versus* SKBR3-ctrl) and 10 times faster than cells expressing cadherin-11 alone ($P < 0.001$, SKBR3-cad11+var *versus* SKBR3-cad11). MCF7 cells expressing cadherin-11 and variant (MCF7-cad11+var) were approximately 5 times more invasive than MCF7 control cells (MCF7-ctrl; $P < 0.05$). Other experiments (data not shown) indicate that all MCF7-cad11+var clones exhibit a similar increase in invasive activity. *Error bars* represent SD over 3 wells/experiment.

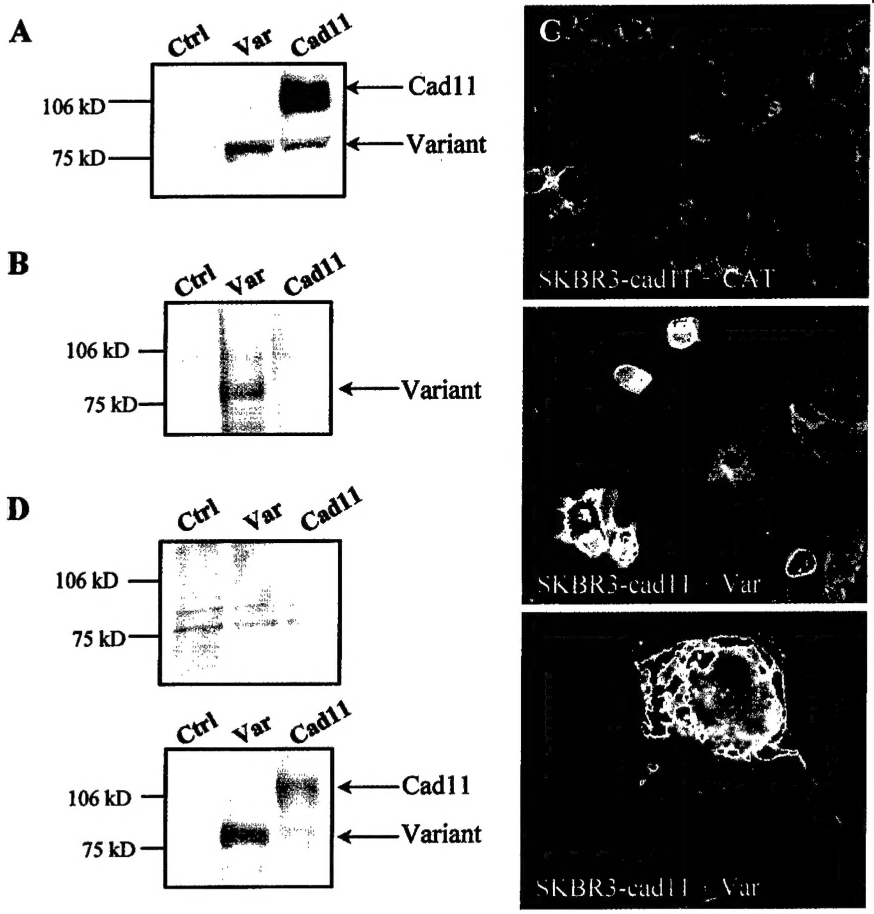

Fig. 8. *A*, MCF7 parental cells were transiently transfected with cDNA encoding CAT (control), cadherin-11, or variant. Twenty-four h later, cells were incubated at 22°C for an additional 24 h before lysis in sample buffer. Whole cell lysates (LDS) were Western blotted and incubated with an antibody that recognizes both the wild-type and variant forms. Lysate from the variant lane (*Var*) exhibits a strong band at approximately M_r 80,000 (*arrow*). There is no band at M_r 120,000 corresponding to wild-type cadherin-11. The cadherin-11 lane (*Cad11*) does exhibit a strong M_r 120,000 band as well as an additional band at approximately M_r 80,000, a presumptive breakdown product. *B*, NP40-insoluble fractions were isolated from cells transiently transfected with the variant or wild-type cadherin-11 cDNA and analyzed by Western blot. The variant remains in the insoluble fraction, which contains cytoskeletal elements and associated proteins, as well as extracellular matrix components. *C*, SKBR3 cells stably expressing wild-type cadherin-11 were transiently transfected with cDNA encoding the variant or CAT (control). Twenty-four h after transfection, cells were trypsinized, plated on coverslips, and subsequently incubated at 22°C for another 24 h to reduce protein secretion. Cells were then fixed, permeabilized, and stained using an antibody that recognizes both cadherin-11 and variant proteins. Analysis by confocal microscopy revealed the presence of variant protein in the cytoplasm of SKBR3-cad11+var cells, but not SK-cad11+CAT cells. This expression was so strong it saturated the confocal image (saturation is indicated by *red* in the field). To accommodate this, we decreased the sensitivity threshold when visualizing the SKBR3-cad+var cells. Consequently, the membrane staining appears to be decreased. *D*, MCF7 cells were transiently transfected with CAT, cadherin-11, or variant cDNA and placed in low-serum media to determine whether the variant protein might be secreted. Media were collected 72 h after transfection and concentrated 40-fold in the presence of protease inhibitors. Western blot analysis of conditioned media failed to detect the variant (*top blot*). Whole cell lysates of transfected cells verify the presence of the variant protein (*bottom blot*).

normally exhibit barely detectable levels of the protein. This is further proven by analysis of β-catenin signaling in these cells in both the absence and presence of cadherin-11. These data represent the first indication that cadherin-11 can act as a functional adherens junction molecule, actively recruiting catenins to a membrane complex and, in the case of β-catenin, stabilizing its expression and affecting its signaling function.

In addition, we found that cadherin-11 can interact with p120^{ctn}. Thoreson *et al.* (22) showed previously that p120^{ctn} did not associate with membrane complexes in MDA-MB-231 cells, which only express cadherin-11, suggesting that cadherin-11 and p120^{ctn} could not interact. Although we also found that p120^{ctn} was not present at the membrane of MDA-MB-231 cells, our experiments clearly demonstrate that cadherin-11 can form complexes with the catenins in general and with p120^{ctn} in particular. This finding is consistent with the fact that the p120^{ctn}-binding region of cadherin-11 is almost perfectly conserved with that of the other cadherins. The failure of cadherin-11-expressing MDA-MB-231 cells to recruit p120^{ctn} to the membrane must therefore be a consequence of some other aspect of their transformed phenotype. Elucidation of the mechanism responsible for the lack of interaction between cadherin-11 and p120^{ctn} might even explain in part the aggressive phenotype of this cell line because p120^{ctn} interaction with cadherins has previously been associated with regulation of adhesion (28).

Effects of Cadherin-11 on Breast Cancer Cell Proliferation, Anchorage-independent Growth, and Invasion. After establishing that cadherin-11 was capable of interacting with catenins and forming an apparently functional adherens junction, we wished to determine whether its expression could alter the phenotype of breast cancer cell lines by assaying proliferation rate, anchorage-independent growth, and invasive activity. Expression of cadherin-11 in SKBR3 cells increased their proliferation rate modestly compared with control cells. Concomitant expression of variant resulted in cells whose proliferation rate was modestly reduced compared with control. Although the difference between the two cell populations was significant, it is important to note that the overall change in proliferation was fairly small. These findings suggest that cadherin-11 alone can act as a mitogenic factor in SKBR3 cells, likely by establishing adhesive contacts between cells. Moreover, no consistent changes in proliferation were observed in cadherin-11-expressing MCF7 cells. Examination of anchorage-independent growth in the presence of exogenous cadherin-11 with or without the variant revealed no significant changes in either SKBR3 cells or MCF7 cells.

Finally, we investigated the ability of cadherin-11 to induce changes in the invasive capacity of both epithelial cell lines. In keeping with its ability to promote formation of the adherens junction, expression of cadherin-11 markedly reduced the invasive activity of SKBR3 cells. Remarkably, concomitant expression of the variant protein led to cells that were 2.5 times more invasive than control and 10 times more invasive than those expressing cadherin-11 alone. In E-cadherin-expressing MCF7 cells, coexpression of cadherin-11 and variant increased invasion 7-fold. In an earlier study (24), expression of cadherin-11 in the E- and P-cadherin-expressing BT20 cells also led to an increase in their invasive capacity, although it is not clear if both cadherin-11 and its variant form were transfected in this experiment.

As discussed earlier, in all systems thus far examined, both cadherin-11 and its variant form are coexpressed. Nevertheless, changes in the relative amounts of the two forms of cadherin-11 do occur; an increase in the relative amount of the variant form is associated with more aggressive osteosarcoma cells (13). Taken to-

gether, these studies indicate that even though the splice variant does not directly alter the ability of cadherin-11 to assemble junctional complexes, under appropriate circumstances (*e.g.*, a migratory stimulus) it can allow cells to become more invasive. The fact that cadherin-11 and the variant are always coexpressed strongly suggests that both are necessary for the observed functional changes. Finally, cadherin-11- and N-cadherin-induced changes in invasive activity occur regardless of the presence of E-cadherin, indicating that the invasion-suppressing function of E-cadherin is not always predominant (24). Additional studies to identify the mechanisms whereby cadherin-11 and the variant act may shed light on the means by which cancer cells acquire a more invasive phenotype.

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REFERENCES

1. Yagi, T., and Takeichi, M. Cadherin superfamily genes: functions, genomic organization, and neurologic diversity. Genes Dev., *14:* 1169–1180, 2000.
2. Yap, A. S., Brieher, W. M., and Gumbiner, B. M. Molecular and functional analysis of cadherin-based adherens junctions. Annu. Rev. Cell Dev. Biol., *13:* 119–146, 1997.
3. Troyanovsky, S. M. Mechanism of cell-cell adhesion complex assembly. Curr. Opin. Cell Biol., *11:* 561–566, 1999.
4. Provost, E., and Rimm, D. L. Controversies at the cytoplasmic face of the cadherin-based adhesion complex. Curr. Opin. Cell Biol., *11:* 567–572, 1999.
5. Steinberg, M. S., and McNutt, P. M. Cadherins and their connections: adhesion junctions have broader functions. Curr. Opin. Cell Biol., *11:* 554–560, 1999.
6. McNeill, H., Ozawa, M., Kemler, R., and Nelson, W. J. Novel function of the cell adhesion molecule uvomorulin as an inducer of cell surface polarity. Cell, *62:* 309–316, 1990.
7. Hermiston, M. L., Wong, M. H., and Gordon, J. I. Forced expression of E-cadherin in the mouse intestinal epithelium slows cell migration and provides evidence for non-autonomous regulation of cell fate in a self renewing system. Genes Dev., *10:* 985–996, 1996.
8. Ji, X., Woodard, A. S., Rimm, D. L., and Fearon, E. R. Transcriptional defects underlie loss of E-cadherin expression in breast cancer. Cell Growth Differ., *8:* 773–778, 1997.
9. Hazan, R. B., Phillips, G. R., Qiao, R. F., Norton, L., and Aaronson, S. A. Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis [published erratum appears in J. Cell Biol., *149:* 236, 2000]. J. Cell Biol., *148*: 779–790, 2000.
10. Peralta, S. A., Knudsen, K. A., Salazar, H., Han, A. C., and Keshgegian, A. A. P-cadherin expression in breast carcinoma indicates poor survival. Cancer (Phila.), *86:* 1263–1272, 1999.
11. Okazaki, M., Takeshita, S., Kawai, S., Kikuno, R., Tsujimura, A., Kudo, A., and Amann, E. Molecular cloning and characterization of OB-cadherin, a new member of cadherin family expressed in osteoblasts. J Biol. Chem., *269:* 12092–12098, 1994.
12. Hoffmann, I., and Balling, R. Cloning and expression analysis of a novel mesodermally expressed cadherin. Dev. Biol., *169*: 337–346, 1995.
13. Kawaguchi, J., Takeshita, S., Kashima, T., Imai, T., Machinami, R., and Kudo, A. Expression and function of the splice variant of the human cadherin-11 gene in subordination to intact cadherin-11. J. Bone Miner. Res., *14:* 764–775, 1999.
14. Pishvaian, M. J., Feltes, C. M., Thompson, P., Bussemakers, M. J., Schalken, J. A., and Byers, S. W. Cadherin-11 is expressed in invasive breast cancer cell lines. Cancer Res., *59:* 947–952, 1999.
15. Kashima, T., Kawaguchi, J., Takeshita, S., Kuroda, M., Takanashi, M., Horiuchi, H., Imamura, T., Ishikawa, Y., Ishida, T., Mori, S., Machinami, R., and Kudo, A. Anomalous cadherin expression in osteosarcoma. Possible relationships to metastasis and morphogenesis. Am. J. Pathol., *155:* 1549–1555, 1999.
16. Munro, S. B., Turner, I. M., Farookhi, R., Blaschuk, O. W., and Jothy, S. E-cadherin and OB-cadherin mRNA levels in normal human colon and colon carcinoma. Exp. Mol. Pathol., *62:* 118–122, 1995.
17. Shibata, T., Ochiai, A., Gotoh, M., Machinami, R., and Hirohashi, S. Simultaneous expression of cadherin-11 in signet-ring cell carcinoma and stromal cells of diffuse type gastric cancer. Cancer Lett., *99:* 147–153, 1996.
18. Shimazui, T., Giroldi, L. A., Bringuier, P. P., Oosterwijk, E., and Schlaken, J. A. Complex cadherin expression in renal cell carcinoma. Cancer Res., *56:* 3234–3237, 1996.
19. Orford, K., Orford, C. C., and Byers, S. W. Exogenous expression of β-catenin regulates contact inhibition, anchorage-independent growth, anoikis and radiation-induced cell-cycle arrest. J. Cell Biol., *146:* 1–13, 1999.
20. Sommers, C. L., Byers, S. W., Thompson, E. W., Torri, J. A., and Gelmann, E. P. Differentiation state and invasiveness of human breast cancer cell lines. Breast Cancer Res. Treat., *31:* 325–335, 1994.
21. Pierceall, W. E., Woodard, A. S., Morrow, J. S., Rimm, D., and Fearon, E. R. Frequent alterations in E-cadherin and α and β catenin expression in human breast cancer cell lines. Oncogene, *11:* 1319–1326, 1995.
22. Thoreson, M. A., Anastasiadis, P. Z., Daniel, J. M., Ireton, R. C., Wheelock, M. J., Johnson, K. R., Hummingbird, D. K., and Reynolds, A. B. Selective uncoupling of p120^{ctn} from E-cadherin disrupts strong adhesion. J. Cell Biol., *148:* 189–202, 2000.
23. Gottardi, C. J., Wong, E., and Gumbiner, B. M. E-cadherin suppresses cellular transformation by inhibiting β-catenin signaling in an adhesion-independent manner. J. Cell Biol., *153:* 1049–1060, 2001.
24. Nieman, M. T., Prudoff, R. S., Johnson, K. R., and Wheelock, M. J. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J. Cell Biol., *147:* 631–644, 1999.
25. Alberts, B. D. Molecular Biology of the Cell. New York: Garland Publishing, Inc., 1994.
26. Wyke, J. A. Overview: burgeoning promise in metastasis research. Eur. J. Cancer, *36:* 1589–1594, 2000.
27. Perl, A. K., Wilgenbus, P., Dahl, U., Semb, H., and Christofori, G. A causal role for E-cadherin in the transition from adenoma to carcinoma. Nature (Lond.), *392:* 190–193, 1998.
28. Yap, A. S., Niessen, C. M., and Gumbiner, B. M. The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120^{ctn}. J. Cell Biol., *141:* 779–789, 1998.